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Molecular Characterization of the Skin Fungal Microbiota in Patients with Seborrheic Dermatitis

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Abstract

Background: Seborrheic dermatitis (SD) is an inflammatory disease associated with seborrhea that appears most often on the face, especially the nasolabial folds, scalp, and upper trunk, in sebaceous gland-rich areas of the skin. Although various factors are involved in the development of SD, Malassezia species of skin fungi play an important role in this skin disease. The aim of this study was to obtain basic information to elucidate the mycological involvement in the development of SD by performing a comprehensive analysis of the skin fungal microbiota of lesional and non-lesional sites in SD patients using a pyrosequencing approach.

Methods: Scale samples were collected from lesional and non-lesional sites of 24 Japanese patients with seborrheic dermatitis and the skin fungal microbiome in the scale samples was analyzed using a pyrosequencing method.

Results: Thirty taxa were detected from a total of 480,186 high-quality sequences. Malassezia species predominated at both lesional and non-lesional sites; however, the fungal microbiota at lesional sites was more diverse than at non-lesional sites. The fungal communities at lesional and non-lesional sites were clearly distinguished on principal coordinate analysis.

Conclusion: Our data indicated that the skin fungal microbiota in lesional sites of SD patients was independent of that in non-lesional sites and will be utilized as basic information for understanding the association between skin fungi and development of SD.

Keywords: Seborrheic dermatitis; Malassezia; Fungi; Pyrosequencing

Abbreviations: SD: Seborrheic Dermatitis; PCoA: Principal Coordinate Analysis; IGS: Intergenic Spacer

Introduction

Seborrheic dermatitis (SD) is a common inflammatory skin disorder associated with seborrhea. It is characterized by erythematous patches with yellow-gray scales. These appear most often on the face, especially the nasolabial folds, scalp, and upper trunk, in sebaceous gland-rich areas of the skin. The disorder is present in about 3% of the general population, more prevalent in males than in females, and frequently observed in patients with acquired immunodeficiency syndrome (AIDS) and Parkinson's disease [1,2]. The disease presents in two age groups: newborn infants up to 5 months of age, and young adults with increased sebum secretion from the skin.

The human skin is populated by various microorganisms, including bacteria and fungi [3,4]. Of these, skin fungi, *Malassezia* species, play an important role in the development of SD. As *Malassezia* species require fatty acids for their growth, they colonize the sebaceous gland-rich areas of the skin, including the face, scalp, and back, where they feed on fatty acids from human sebum. *Malassezia* species secrete lipases that hydrolyze sebum into triglycerides, which are further hydrolyzed into fatty acids [5-7]. Fatty acids are utilized as nutrition by skin microorganisms, including *Malassezia*. However, the unsaturated fatty acid oleic acid causes inflammation of the skin directly by eliciting IL-1α secretion from macrophages or epidermal keratinocytes, and is thought to be the causative agent of SD [7]. In fact, the mRNA expression of a specific *Malassezia* lipase gene can be detected from lesional sites in patients with SD [8,9]. The clinical condition improves on administering antifungal agents, suggesting that *Malassezia* species are one of the causative agents of SD [10,11].

Tajima et al. [12] analyzed the *Malassezia* microbiota in the skin of patients with SD using a DNA-based molecular approach. The genus *Malassezia* currently includes 14 species; of these, *M. globosa* and *M. restricta* are the major species in the skin of patients with SD and these species are more abundant at lesional sites than non-lesional sites.

In the present study, we analyzed comprehensively the skin fungal microbiota of lesional and non-lesional sites of SD patients to obtain basic information to enable understanding of the development of SD and skin fungal microbiota using a pyrosequencing approach.

Materials and Methods

Patients and sample collection

Twenty-four Japanese outpatients with SD were enrolled in this study (19 males and 5 females; mean age 47.8 ± 18.8 (range 20-77) years). Patients who took antimicrobial agents before involvement in this study were excluded. The study protocol was approved by our Institutional Review Board and informed consent was obtained from each individual.

Skin fungi were collected by applying a 5×7 cm OpSite™ transparent adhesive dressing (Smith and Nephew Medical, Hull, UK)

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using the method of Sugita et al. [13]. Briefly, dressings were applied three times each to both lesional and non-lesional sites on the faces of SD patients.

Fungal DNA extraction

The DNA was extracted directly from the dressing using the method of Sugita et al. [13]. Briefly, the dressing was placed in a 1.5 mL Eppendorf tube with 1 mL of lysing solution (100 mM Tris–HCl (pH 8.0), 30 mM EDTA (pH 8.0), and 0.5% SDS) and incubated for 15 minutes at 100°C. The suspension was extracted with phenol–chloroform–isoamyl alcohol (25:24:1, vol/vol/vol), and subsequently, chloroform–isoamyl alcohol (24:1, vol/vol). The DNA was precipitated with 2.5 volumes of ethanol, in the presence of 3 M sodium acetate and Ethachinmate (Nippon Gene, Toyama, Japan), according to the manufacturer's instructions.

Pyrosequencing

The universal primers NL1 and NL4 containing the A and B sequencing adaptors were used for PCR amplification of the D1/D2 variable regions of the 26S rRNA gene. The primers used were B-NL1 (5'-cctatcccctgtgtgccttggcagtctcaGCATATCAATAAGCGGAGGAAAAG-3'; adaptor B in lowercase) and A-NL4 (5'-ccatctcatccctgcgtgtctccgactcagatcagacacgNNNNNGGTCCGTGTTTCAAGACGG-3'; adaptor A in lowercase letter, N represents a bar code unique to each sample). The cycling conditions were 94°C for 1 min, followed by 30 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 10 min. A negative control containing no template was also used. Following purification of each PCR amplicon, equimolar amounts of each PCR reaction were mixed in a single tube. Then, the purified amplicon mixtures were sequenced in a 454 GS FLX pyrosequencing platform (Roche Diagnostics Japan, Tokyo, Japan), according to the manufacturer's instructions.

Sequence processing and data analysis

The primer and barcode sequences were removed from the data and possible chimeras were also excluded from the analysis. Sequences ≥400 bp and <1,000 bp in length were subjected to analysis. The D1/D2 LSU sequences were classified to the genus level using the RDP classifier (http://rdp.cme.msu.edu/). The D1/D2 LSU sequence of the type strains of yeast-like fungi was extracted from GenBank. The sequences were classified to the species level using an in-house BLAST search. The R package vegan [http:/CRAN.R-project.org/package=vegan] was used to construct the Shannon diversity index boxplot. Significance was tested using a *t*-test with a one-tailed distribution and two-sample variance. A three-dimensional principal coordinate analysis (PCoA) plot was normalized using weighed values (http://www.quiime.org).

Results

Fungal community composition

This study analyzed 480,186 high-quality sequences ≥400 bp and <1,000 bp in length, ranging from 6,058 to 13,773 reads per sample. The numbers of reads for lesional and non-lesional sites did not differ significantly (lesional sites 9,744 ± 1,864, non-lesional sites 10,263 ± 2,044). The samples were grouped into 30 genera in two phyla: ten yeast-like Ascomycota and 20 filamentous Basidiomycota (Table 1). *Malassezia* was the most abundant fungus genus at both sites, and predominated at non-lesional sites (83.3 ± 26.2%) compared with lesional sites (57.3 ± 9.6%) (Supplement Figure 1), while the proportion

Phylogenetic group	Taxa	Lesional site		Non-lesional site		Phylogenetic group	Taxa	Lesional site		Non-lesional site	
		Mean (%)	SD	Mean (%)	SD			Mean (%)	SD	Mean (%)	SD
Filamentous fungi	*Alternaria*	0.41	1.71	0.04	0.15	Non-*Malassezia*	*Candida albicans*	0.39	1.85	0.00	0.00
	Aphanoascus	0.01	0.04	0.00	0.00	yeast-like fungi	*Candida azyma*	0.01	0.02	0.00	0.02
	Aspergillus	1.27	3.41	0.06	0.16		*Candida etchellsii*	0.00	0.01	0.00	0.00
	Aureobasidium	0.07	0.20	0.01	0.02		*Candida sake*	0.01	0.03	0.00	0.02
	Botrytis	0.00	0.00	0.00	0.02		*Candida parapsilosis*	11.04	9.38	1.02	1.59
	Cladosporium	3.07	5.42	1.12	1.77		*Candida guilliermondii*	5.82	7.47	1.12	2.31
	Cucurbita	0.00	0.00	0.00	0.01		*Cryptococcus oeirensis*	0.04	0.20	0.00	0.00
	Cyphellophora	0.15	0.72	0.00	0.00		*Cryptococcus saitoi*	1.36	6.66	0.18	0.53
	Eutypella	0.10	0.47	0.00	0.02		*Cryptococcus albidus*	2.43	5.12	0.64	0.87
	Hexagonia	0.00	0.00	0.00	0.00		*Cryptococcus magnus*	1.65	2.51	0.21	0.43
	Meira	0.00	0.00	0.04	0.11		*Erythrobasidium hasegawianum*	0.00	0.02	0.00	0.00
	Neosetophoma	0.01	0.03	0.00	0.00		*Filobasidium uniguttulatum*	0.51	2.49	0.01	0.04
	Paraconiothyrium	0.00	0.00	0.01	0.02		*Saccharomyces bayanus*	0.00	0.00	0.07	0.25
	Penicillium	0.00	0.00	0.01	0.02		*Tilletiopsis minor*	0.05	0.21	0.05	0.21
	Pestalotiopsis	0.26	1.27	0.00	0.01		*Trichosporon asahii*	0.05	0.25	0.30	1.40
	Phoma	0.35	1.70	0.00	0.00		*Wickerhamomyces anomalus*	0.53	2.56	0.00	0.02
	Plectosphaerella	0.23	1.11	0.00	0.00		*Rhodotorula minuta*	1.47	3.09	0.80	2.08
	Sarcinomyces	0.00	0.02	0.00	0.00		*Rhodotorula mucilaginosa*	11.30	9.66	2.92	3.42
	Thanatephorus	0.00	0.01	0.00	0.00						
	Toxicocladosporium	0.07	0.30	0.08	0.20						
Genus *Malassezia*	*Malassezia restricta*	49.60	9.10	52.88	12.52						
	Malassezia globosa	4.99	3.94	31.02	15.42						
	Malassezia sympodialis	2.63	6.61	4.66	6.28						
	Malassezia furfur	0.12	0.29	0.13	0.27						
	Malassezia slooffiae	0.00	0.01	2.62	7.41						

Table 1: The percentage of fungi detected from scale samples from patients with seborrheic dermatitis.

of non-*Malassezia* yeast-like fungi was higher at lesional sites (36.7 ± 10.7%) than non-lesional sites (7.1 ± 5.6%). Filamentous fungi accounted for 6.0 ± 6.4% and 1.3 ± 1.8% at lesional and non-lesional sites, respectively. Nine taxa accounted for approximately 3% of the sequences at both lesional and non-lesional sites: *Candida parapsilosis* and *C. guilliermondii*, *Cryptococcus albidus*, *Rhodotorula mucilaginosa*, *Malassezia restricta*, *M. globosa*, *M. sympodialis*, and *M. slooffiae*, and *Cladosporium* spp. (Figure 1) and accounted for over approximately 90% of all of the fungal species. The colonization levels of *M. furfur*, *M. sympodialis*, and *M. slooffiae* did not differ significantly between lesional and non-lesional sites, whereas colonization by both *M. globosa* and *M. restricta* differed significantly. *M. restricta* predominated at lesional sites (87.1 ± 11.0%) versus non-lesional sites (58.1 ± 13.9%), while *M. globosa* predominated at non-lesional sites (33.6 ± 15.6%) versus lesional sites (8.4 ± 6.0%) (Supplement Figure 2).

The Shannon diversity index was calculated to determine the

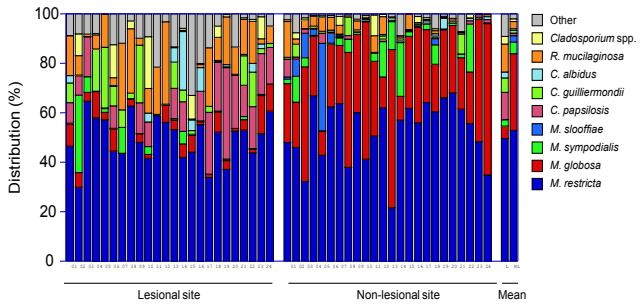

Figure 1: Distribution of the nine major taxa.

The distributions of 9 of the 30 taxa are shown.

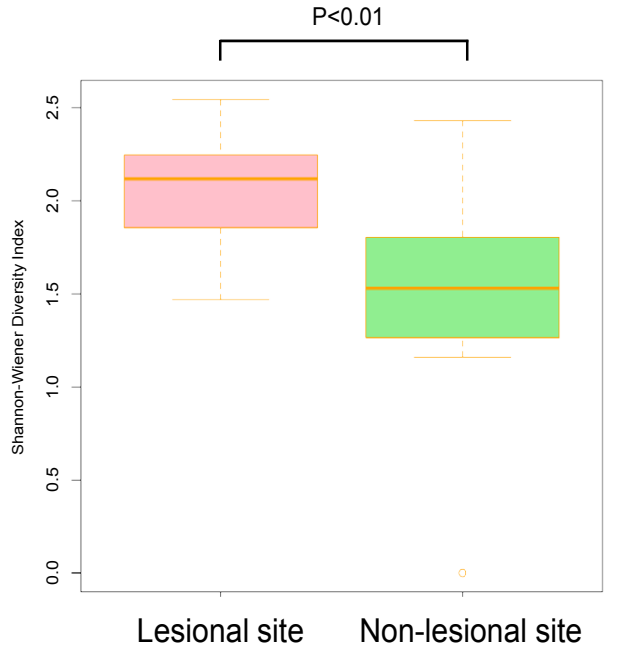

Figure 2: Shannon diversity index.

Lesional sites were significantly (p<0.05) more diverse than non-lesional sites.

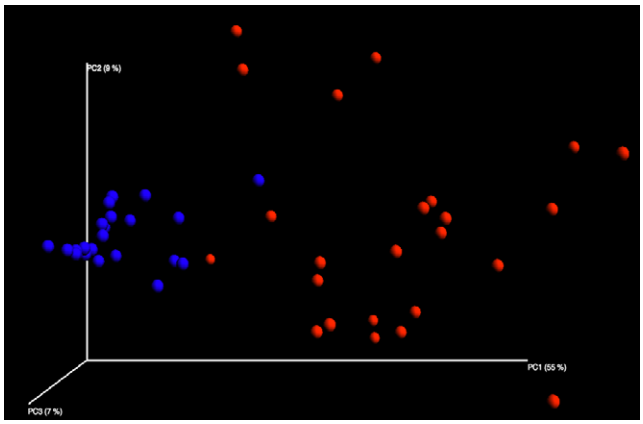

Figure 3: The fungal communities of skin samples obtained from lesional and non-lesional sites.

The fungal communities were generated by principal coordinates analysis (PCoA).

Red: lesional site; blue: non-lesional site.

diversity of the samples. Lesional samples showed significantly greater diversity than non-lesional samples (p<0.01) (Figure 2). PCA was used to evaluate sample diversity and analyze the relationships among samples, and revealed clear separation between lesional and non-lesional sites (Figure 3).

Discussion

This study is the first comprehensive analysis of the skin fungal microbiome of SD patients using a pyrosequencing method.

Studies of the relationship between skin microorganisms and SD have focused on *Malassezia* species, since they secrete lipase to hydrolyze sebum into fatty acids. A molecular-based culture-independent method should be used to analyze the cutaneous *Malassezia* microbiota in healthy subjects or patients as no culture medium that allows efficient recovery of *Malassezia* from scale samples is available. Tajima et al. [12] first investigated the skin *Malassezia* microbiome of SD patients using a culture-independent method. The level of *Malassezia* colonization at lesional sites was approximately threefold that at non-lesional sites. In addition, *M. restricta* predominated over *M. globosa*. *Malassezia* is part of the normal flora in healthy individuals, but is also associated not only with SD but also with pityriasis versicolor and atopic dermatitis [14,15]. Both *M. restricta* and *M. globosa* are major components of various skin diseases, although the ratio of the two is disease-specific [16,17]. In this study, the pyrosequencing assay revealed that *M. restricta* predominated over *M. globosa* at lesional sites (Supplement Figure 2). While the ratio of *M. restricta* to the total *Malassezia* colonization level at non-lesional sites was significantly higher than that at lesional sites, that of *M. restricta* was almost the same at both lesional (49.6%) and non-lesional (52.9%) sites in terms of total fungal colonization level. This was due to the fact that the colonization level of non-*Malassezia* yeast species was higher at lesional sites than at non-lesional sites. Of the 30 taxa identified in this study, 9 accounted for more than 90% of the total colonization. These microorganisms are normal fungal flora in healthy subjects, although *Candida parapsilosis*, *C. guilliermondii*, *Cryptococcus albidus*, and *Rhodotorula mucilaginosa* rarely cause opportunistic fungal infections in immunocompromised hosts [3,18-20].

The greater quantity of sebum at lesional sites than non-lesional

sites is thought to explain why the fungal microbiota was more diverse or greater fungal colonization was present at lesional sites. Since *Malassezia* species require fatty acids for their growth, they have more lipase genes than other skin fungi, with *M. globosa* and *M. restricta* possessing 15 and 10 lipase genes (unpublished data), respectively. *Candida* species also secrete lipases, so lipases secreted by other fungi might act synergistically in the development of seborrheic dermatitis [9,21].

Bacteria and other fungi might also be involved in the development of seborrheic dermatitis. The fungal microbiomes of the scalps of patients with and without dandruff are similar, whereas the bacterial microbiomes are different [22]. *Staphylococcus epidermidis* predominated on the dandruff scalps (60%), while *Propionibacterium acnes* predominated on normal scalps (74%) in French subjects. A discrepancy in the fungal microbiome was observed between that French study and a Korean report. The latter study reported that *Filobasidium* species were most common in dandruff patients, while *Acremonium* species were most common in healthy subjects. Surprisingly, no *Malassezia* species were detected from the scalp samples; this may have been due to differences in the techniques used [23].

The genotype of *Malassezia* species might also be involved in the development of seborrheic dermatitis. As the intergenic spacer (IGS) region of the *Malassezia* rRNA gene shows remarkable intra-species diversity, population structure can be elucidated by genotypic analyses. Tajima et al. [12] and Hiruma et al. [24] found patient-specific IGS genotypes of *M. globosa* and *M. restricta* in patients with seborrheic dermatitis and dandruff.

In conclusion, this study found that the fungal communities were more diverse at lesional sites than non-lesional sites. Our findings provide useful information for understanding the interactions between skin microorganisms and seborrheic dermatitis.

References

1. Ashbee HR, Evans EG (2002) Immunology of diseases associated with Malassezia species. Clin Microbiol Rev 15: 21-57.
2. Gupta AK, Batra R, Bluhm R, Boekhout T, Dawson TL Jr (2004) Skin diseases associated with Malassezia species. J Am Acad Dermatol 51: 785-798.
3. Findley K, Oh J, Yang J, Conlan S, Deming C, et al. (2013) Topographic diversity of fungal and bacterial communities in human skin. Nature 498: 367-370.
4. Grice EA, Segre JA (2011) The skin microbiome. Nat Rev Microbiol 9: 244-253.
5. Ro BI, Dawson TL (2005) The role of sebaceous gland activity and scalp microfloral metabolism in the etiology of seborrheic dermatitis and dandruff. J Investig Dermatol Symp Proc 10: 194-197.
6. DeAngelis YM, Gemmer CM, Kaczvinsky JR, Kenneally DC, Schwartz JR, et al. (2005) Three etiologic facets of dandruff and seborrheic dermatitis: Malassezia fungi, sebaceous lipids, and individual sensitivity. J Investig Dermatol Symp Proc 10: 295-297.
7. James AG, Abraham KH, Cox DS, Moore AE, Pople JE (2013) Metabolic analysis of the cutaneous fungi Malassezia globosa and M. restricta for insights on scalp condition and dandruff. Int J Cosmet Sci 35: 169-175.
8. Lee YW, Lee SY, Lee Y, Jung WH (2013) Evaluation of Expression of Lipases and Phospholipases of Malassezia restricta in Patients with Seborrheic Dermatitis. Ann Dermatol 25: 310-314.
9. Xu J, Saunders CW, Hu P, Grant RA, Boekhout T, et al. (2007) Dandruff-associated Malassezia genomes reveal convergent and divergent virulence traits shared with plant and human fungal pathogens. Proc Natl Acad Sci USA 104: 18730-18735.
10. Faergemann J, Borgers M, Degreef H (2007) A new ketoconazole topical gel formulation in seborrhoeic dermatitis: an updated review of the mechanism. Expert Opin Pharmacother 8: 1365-1371.
11. Swinyer LJ, Decroix J, Langner A, Quiring JN, Blockhuys S (2007) Ketoconazole gel 2% in the treatment of moderate to severe seborrheic dermatitis. Cutis 79: 475-482.
12. Tajima M, Sugita T, Nishikawa A, Tsuboi R (2008) Molecular analysis of Malassezia microflora in seborrheic dermatitis patients: comparison with other diseases and healthy subjects. J Invest Dermatol 128: 345-351.
13. Sugita T, Suto H, Unno T, Tsuboi R, Ogawa H, et al. (2001) Molecular analysis of Malassezia microflora on the skin of atopic dermatitis patients and healthy subjects. J Clin Microbiol 39: 3486-3490.
14. Ashbee HR (2007) Update on the genus Malassezia. Med Mycol 45: 287-303.
15. Gaitanis G, Magiatis P, Hantschke M, Bassukas ID, Velegraki A (2012) The Malassezia genus in skin and systemic diseases. Clin Microbiol Rev 25: 106-141.
16. Sugita T, Zhang E, Tanaka T, Nishikawa A, Tajima M, et al. (2013) Recent advances in research on Malassezia microbiota in humans. Med Mycol J 54: 39-44.
17. Sugita T, Zhang E, Tanaka T, Tajima M, Tsuboi R, et al. (2012) Atopic Dermatitis and Skin Fungal Microorganisms, In Atopic Dermatitis-Disease Etiology and Clinical Management, Dr. Jorge Esparza-Gordillo (Eds.), InTech Open Access.
18. Zhang E, Tanaka T, Tajima M, Tsuboi R, Nishikawa A, et al. (2011) Characterization of the skin fungal microbiota in patients with atopic dermatitis and in healthy subjects. Microbiol Immunol 55: 625-632.
19. Miceli MH, Díaz JA, Lee SA (2011) Emerging opportunistic yeast infections. Lancet Infect Dis 11: 142-151.
20. Groll AH, Walsh TJ (2001) Uncommon opportunistic fungi: new nosocomial threats. Clin Microbiol Infect 7 Suppl 2: 8-24.
21. Park M, Do E, Jung WH (2013) Lipolytic enzymes involved in the virulence of human pathogenic fungi. Mycobiology 41: 67-72.
22. Clavaud C, Jourdain R, Bar-Hen A, Tichit M, Bouchier C, et al. (2013) Dandruff is associated with disequilibrium in the proportion of the major bacterial and fungal populations colonizing the scalp. PLoS One 8: 58203.
23. Park HK, Ha MH, Park SG, Kim MN, Kim BJ, et al. (2012) Characterization of the fungal microbiota (mycobiome) in healthy and dandruff-afflicted human scalps. PLoS One 7: e32847.